FEBS Letters 583 (2009) 430–436

journal homepage: www.FEBSLetters.org

Zeb1-mediated T-cadherin repression increases the invasive potential of gallbladder cancer

Yoshihiro Adachi [a], Tamotsu Takeuchi [a,*], Tomoko Nagayama [b], Yuji Ohtsuki [c], Mutsuo Furihata [a]

[a] Department of Pathology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan
[b] JST Innovation Satellite Kochi Practical Application Research, Tosayamada, Japan
[c] Division of Pathology, Matsuyama Shimin Hospital, Matsuyama, Japan

ARTICLE INFO

Article history:
Received 1 October 2008
Revised 4 December 2008
Accepted 18 December 2008
Available online 29 December 2008

Edited by Beat Imhof

Keywords:
Gallbladder cancer
T-cadherin
Zeb1
Epithelial-mesenchymal transition
Cancer invasion

ABSTRACT

Here, we report that the transcriptional regulator Zeb1 repressed the transcription of T-cadherin, to increase the invasive activity of gallbladder cancer cells. Zeb1 physically bound to the promoter of T-cadherin, repressed promoter activity in E-box-like sequence-dependent fashion, and suppressed T-cadherin expression. In gallbladder cancer tissues, Zeb1 was expressed at the cancer invasion front, whereas T-cadherin was exclusively expressed in non-invasive foci. Collagen gel invasion assay showed that T-cadherin was a negative regulator for gallbladder cancer invasion. These findings suggest that Zeb1 represses T-cadherin expression and thus increases the invasive activity of gallbladder cancer.

1. Introduction

Gallbladder cancer is an aggressive tumor with strong invasive activity into adjacent organs. It is well established that depth of cancer invasion is strongly correlated with prognosis in patients with gallbladder cancer [1]. Unfortunately, it is often difficult to cure advanced invasive gallbladder cancer with conventional treatment.

Genotoxic stress is, in association with long-term inflammation, important in the tumorigenesis of gallbladder cancer [2,3]. Studies focused on molecular mechanisms of tumorigenesis have revealed mutations of p53 [4–6], activating mutations of the KRAS proto-oncogene [7], and loss of cell-cycle regulation by CDK-INK4A [8] in gallbladder cancer. However, the molecular mechanisms involved in gallbladder cancer invasion remain unclear.

Recent studies have highlighted epithelial-mesenchymal transition (EMT) as a crucial process in cancer invasion and metastasis [9,10]. Cancer cells acquire fibroblast-like properties by EMT as well as increased motility. In the process of EMT, transcription factors such as Snails, bHLH, and Zebs repress the expression of various epithelial cell-related genes including E-cadherin while up-regulating the expression of mesenchymal cell-related genes [11,12].

T-cadherin (also referred to as CDH13 or H-cadherin) is a glycosyl phosphatidyl inositol (GPI)-linked surface membrane protein [13], and is expressed in various epithelial cells [14,15]. T-cadherin lacks a cytoplasmic domain, which is required for the homophilic binding activity of classical cadherins [16]. Moreover, a tryptophan conserved in all other cadherins and which plays a crucial role in adhesive function is replaced by an isoleucine in T-cadherin. Given these unique molecular structural features, and its localization on the lipid raft, which partitions various signaling molecules, T-cadherin is thought to play roles in signal transduction apart from cell-cell adhesion [17–20]. Consistent with this, NMR analysis very recently demonstrated that T-cadherin exhibits little structural evidence of homophilic adhesive activity [21].

Interestingly, many epigenetic studies focused on analysis of promoter methylation have suggested that T-cadherin acts as a tumor suppressor factor in various malignant tumors including those of breast, lung, colon, and skin [14,22–25]. We have examined the pathobiological properties of T-cadherin in various cancers, and noted the presence of a typical E-box-like (also designated as E2-box) element, 5′-CACCTG-3′ [26], to which Zeb1 could bind, in the promoter region of T-cadherin [24,27].

Here, we report that Zeb1 repressed the transcription of T-cadherin and down-regulation of T-cadherin increased cancer invasion activity in gallbladder cancer cell. We think that Zeb1-mediated repression of T-cadherin may be involved in gallbladder cancer invasion. Since restoration of T-cadherin in cultured

* Corresponding author.
E-mail address: takeutit@kochi-u.ac.jp (T. Takeuchi).

0014-5793/$34.00
doi:10.1016/j.febslet.2008.12.042

gallbladder cancer cells markedly decreased cancer invasion activity, T-cadherin could be a possible target for corrective gene therapy.

2. Materials and methods

2.1. Antibodies

A rabbit polyclonal antibody to human Zeb1 was prepared in our laboratory using the methods of Darling et al. with modifications [28,29]. Briefly, PCR product encoding a part of human Zeb1 (amino acids 559–663) was subcloned into the PET16b vector (Novagen Inc., Madison, WI) and confirmed by sequence analysis. Recombinant histidine-tagged protein was obtained using transformed BL21 (DE3) pLyS cells by isopropyl-1-thio-beta-D-galactopyranoside induction, subsequently purified with a Ni-NTA Spin kit (Qiagen, Valencia, CA), and used for immunization of rabbits. Recombinant GST-fused Zeb1 (amino acids 559–663) protein was also obtained using the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ) and used for affinity-column preparation. The specificity of affinity-purified antibody was confirmed by Western-immunoblotting and immunohistochemical staining using well characterized Zeb-1-expressing uterine carcinosarcoma tissue (Supplementary Fig. S1). Details of the procedures used to prepare affinity-purified rabbit specific antibody to human T-cadherin have been described previously [14,17,25]. Antibodies to MHC class I and vimentin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse immunoglobulin antibodies to N-cadherin were purchased from Zymed Laboratories Inc. (San Francisco, CA) and E-cadherin from Takara Bio Inc. (Shiga, Japan). Normal control immunoglobulin was prepared in our laboratory.

2.2. Chromatin immunoprecipitation (ChIP) analysis

Zeb1-expressing but T-cadherin-negative NOZ cells were cross-linked with 1% formaldehyde for 10 min. Cells were suspended in 0.2 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0) for 10 min. Genomic DNA was fragmented to lengths of 200–1000 bp by sonication and diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl). One-tenth of the sample was set aside as an input control, and the remainder was pre-cleared with salmon sperm DNA protein A-Sepharose beads for 30 min with agitation and subsequently incubated with rabbit polyclonal anti-Zeb1 antibody or normal anti-rabbit IgG as a negative control overnight at 4 °C. Immune complexes were pulled down with salmon sperm DNA protein A-Sepharose beads, eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$), and incubated with 5 M NaCl for 4 h at 65 °C to reverse cross-linking. The elutes were treated with proteinase K, and released DNA was purified and amplified by PCR with primers specific for the T-cadherin promoter (sense, 5′-TAC TGC TGT TCT GAG TAC CTG-3′/62220–62200; antisense, 5′-GTG GCC CAA GTC ATG ATG GTG-3′/61955–61975 of Homo sapiens chromosome 16 clone RP11-2L4).

2.3. Electrophoretic mobility shift assay (EMSA)

The entire coding region of human Zeb1 cDNA was generated by RT-PCR with SpeedStar polymerase (Takara) with the sense primer 5′-GCCATGGCGGATGGCCCCAGGTGTAAG-3′ and anti-sense primer 5′-TTAGGCTTCATTTGTCTTTTCTTCAGAC-3′, subsequently ligated into pTarget-T expression vector (Promega, Madison, WI), and confirmed by sequencing. Transfection into Cos7 cells was carried out as described previously [28]. Nuclear lysates were prepared from Cos7 cells, which were transfected with Zeb1-containing pTarget-T.

EMSA was performed using the digoxigenin Gel Shift kit according to the manufacture's protocol (Roche Applied Science, Mannheim, Germany). Briefly, a double-strand probe (−683 to −602) containing the wild E2-box 5′-CACCTG-3′, or replaced by 5′-AAATTT-3′ sequences, were end-labeled with digoxigenin-11-ddUTP. Labeled probes were added to nuclear extract, and the binding reaction was allowed to proceed for 15 min at room temperature. Unlabeled competitor at 50-fold molar excess, or antibodies, anti-Zeb1 or control antibody were added to the binding reactions 5 min before the labeled probe and allowed to incubate at room temperature.

The samples were resolved through a non-denaturing 8% acrylamide gel for separating complexes, subsequently transferred to a nylon membrane using electroblotter, and further handled for immunochemical detection as outlined by the manufacturer.

2.4. Repression of T-cadherin promoter activity by Zeb1

The procedure used for evaluation of promoter activity using the GreatEscApe™SEAP system (Takara) was described in detail previously [28]. The T-cadherin promoter sequence from −1 to −682 was cloned into pSEAP2-Basic plasmid (Takara) between BamH1 and EcoR1 restriction sites, and subsequently confirmed by sequencing. Wild-type and E2-box-mutated promoters were generated by PCR with different 5′ primers and a fixed 3′ primer, using human genomic DNA as template. The sequence of the single reverse primer was 5′-GGA ATT CAT TTT GTC CGA CTA GAA GCG-3′ (−1 to −18), while the forward primers were 5′-AGG ATC CAG ACT CTC ACC TGA GCA GTT-3′ (−663 to −682; E2-box underlined) and 5′-AGG ATC CAG ACT CTA AAT TTA GCA GTT-3′ (E2-box sequence replaced by underlined sequence) for wild-type and E2-box-mutated sequences, respectively.

Wild-type, E2-box-mutated T-cadherin promoter-containing pSEAP2-Basic vectors, or empty vector, and Zeb1-containing pTarget-T vectors were co-transfected into Cos7 cells using DEAE-Dextran. Promoter activity was measured using a GreatEscApe™SEAP chemiluminescent detection kit (Takara). Reporter assays was also performed in the cultured gallbladder cancer cell, OCUG-1, and stably Zeb1 overexpressing OCUG-1 cell, which was described below. In these assays, wild-type, E2-box-mutated T-cadherin promoter-containing pSEAP2-Basic vectors, or empty vector, was transfected.

2.5. Western-immunoblotting

Western-immunoblotting was carried out according to the modified method of Towbin et al. [30], as previously reported [14]. Briefly, equal amounts of proteins were electrophoresed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels and electroblotted to polyvinylidine difluoride membrane (Millipore, Bedford, MA). After blocking with bovine serum albumin, membranes were incubated with antibodies.

2.6. Cell culture

NOZ [31,32] and OCUG-1 [32] gallbladder cancer cells were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). Both NOZ cell and OCUG-1 cells are derived from the malignant peritoneal effusion of patient with gallbladder adenocarcinoma. NOZ cell line composed of spindle and round shaped cells, while OCUG-1 cells proliferated in a monolayer sheet.

Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) (Gibco Life Technologies, NY) containing 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamycin (Gibco RL-Life Technologies, Grand Island, NY).

2.7. Tissue specimens and immunohistochemical staining

Archival pathological tissue specimens were obtained from 30 patients with invasive gallbladder cancer. The 30 gallbladder carcinomas (age 54–76 years; average age 64.6 years; 24 women and 6 men) comprised advanced cases (pT2 10, pT3 12, pT4 8). Of these, eight cases (26.7%) were classified as well-differentiated, 18 cases (60%) as moderately differentiated, and four cases (13.3%) as poorly differentiated adenocarcinomas. TNM staging according to the staging system of the American Joint Committee on Cancer (AJCC) was used [33]. All specimens were surgically obtained, fixed in 10% buffered formalin, and paraffin-embedded. The procedures used for immunohistochemical staining were previously described in detail [14]. In several experiments, antibodies were preadsorbed with antigens to verify specificity.

2.8. siRNA mediated RNA interference

Using the methods of Philippova et al. [34], we employed the siRNA duplexes 5′-GGACCAGUCAAUUCUAAAC-3′ for T-cadherin gene silencing. For *ZEB1* gene silencing, we examined thee siRNA duplexes (Sigma Genosys siRNA Service, St. Louis, MO) and employed 5′-CCCAUUACAGGCAACCAGUTT-3′ (Supplementary Fig. S2). We also used GFP siRNA duplex, with a target sequence 5′-CGGCAAGCUGACCCUGAAGUUCAU-3′, as a non-silencing control. siRNA was transfected into gallbladder cancer cells using Oligofectamine following the manufacturer's instructions (Invitrogen Life Sciences, Carlsbad, CA).

2.9. Tetracycline-induced T-cadherin expression system

The human T-cadherin cDNA sequence was subcloned into pcDNA4/TO/myc-HisA vector (Invitrogen). The resulting vector, designated pcDNA4/TO/T-cad, allowed T-cadherin expression under the control of a tetracycline operator 2 sequence-containing cytomegalovirus promoter. The NOZ cell line was first stably transfected with the pcDNA6/TR vector (Invitrogen), containing sequences encoding the tetracycline repressor protein, using DOTAP transfection reagents (Boehringer Mannheim GmbH, Mannheim, Germany). The bulk population was subsequently transfected with pcDNA4/TO/T-cad plasmid and selected with Zeocin and Blasticidin (Invitrogen). Finally, we established three independent clones which expressed T-cadherin in the presence of 1 μg/ml tetracycline (Supplementary Fig. S3).

2.10. Reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR (TaqMan technology)

RT-PCR was performed as previously described [17]. Briefly, total cellular RNA was prepared from cell lysates using RNA-zol B (Biotex Laboratory, Houston, TX). cDNA synthesis from total RNA and subsequent PCR were performed using an RNA LA (long and accurate) PCR kit (Takara). The procedure was performed according to the manufacturer's protocol. The primer sets used in this study were sense 5′-GCCACGATCATGATCGATGAC-3′ and antisense 5′-GTCTTCATTTTCCACTTTGA-3′ for T-cadherin, sense 5-TTCAGCATCACCAGGCAGTC-3 and antisense 5-GAGTGGAGGAGGCTGAGTAG-3 for Zeb1, and sense 5′-TCCACCACCCTGTTGCTGTA-3′ and antisense 5′-ACCACAGTCCATGCCATCAC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR-amplified products were electrophoretically separated on a 2% agarose gel.

Real-time PCR was also carried out according to the manufacturer's protocols (Prism 7000, Applied Biosystems (ABI), Foster City, CA). The primer/probes Hs 00169908-ml (T-cadherin) and Hs 99999905-ml (*GAPDH*) were purchased from ABI. Levels of expression of T-cadherin were calculated using the comparative cycle time (C_t) value, which was normalized to GAPDH PCR C_t value by subtracting the GAPDH C_t value from the target PCR C_t value to obtain ΔC_t. From ΔC_t, the relative level of mRNA expression to that of GAPDH for T-cadherin was calculated using the following equation: relative mRNA expression = $2^{-(Ct\,\text{target} - Ct\,\text{GAPDH})}$. Real-time PCR was performed in triplicate.

2.11. Cell proliferation and collagen gel invasion assay

Cell proliferation was evaluated by measurement of live cells as previously described [14]. Briefly, 1×10^3 siRNA-treated OCUG-1 cells were cultured on standard 35 mm tissue culture dishes (BD Falcon, San Jose, CA) in triplicate. After 24 and 48 h, live cells were counted.

Collagen gel invasion assay was carried out using collagen solution (type I-A) (Nitta Gelatin, Osaka, Japan). Briefly, 100 μl of collagen gel was set within a 6.5 mm diameter, 8 μm pore filter transwell (Corning Costar, Corning, NY). Then 2×10^4 cells were plated in 100 μl serum-free DMEM, and 1 ml of DMEM supplemented with 10% FCS and recombinant hepatocyte growth factor (final concentration 20 μg/ml) was applied underneath the filter. For tetracycline-induced T-cadherin expression in NOZ cells, 1 μg/ml tetracycline was added to the culture system.

Cells were then allowed to invade out of collagen I matrix across the membrane for 24 and/or 48 h. Non-invasive cells were then removed from the top compartment of the transwell with a cotton swab and the invasive cells present on the underside of the membrane were fixed and stained with hematoxylin. The number of invasive cells was counted per field (×40 microscope objective) from a random field of each membrane.

These assays were performed in triplicate and repeated at least twice.

2.12. Statistical analysis

Statistical analysis was performed by Student's *t*-test for unpaired observation. Findings of $P < 0.01$ were considered significant.

3. Results

3.1. Zeb1 bound and repressed the activity of T-cadherin promoter

We noted that a typical E-box-like sequence (also reported as E2-box motif), 5′-CACCTG-3′, is present in the previously identified T-cadherin promoter. RT-PCR analysis demonstrated that NOZ cells expressed Zeb1, but non-detectable levels of T-cadherin. In contrast, OCUG-1 cells expressed T-cadherin but not Zeb1 (Fig. 1A).

Using NOZ cells, we performed ChIP assay to determine whether the T-cadherin promoter is a direct target of Zeb1. As demonstrated in Fig. 1B, we found a T-cadherin-specific PCR band on immunoprecipitation with anti-Zeb1 antibody in NOZ gallbladder cancer cells. No detectable PCR products were found with control rabbit IgG. The EMSA also indicated that Zeb1 bound to the wild-type T-cadherin promoter containing 5′-CACCTG-3′ sequence, but not that with mutated 5′-AAATTT-3′ sequence (Fig. 1C). These findings suggested that endogenously expressed Zeb1 bound to T-cadherin promoter in the NOZ gallbladder cancer cell.

Because Zeb1 acts not only as a repressor but also as an activator for several genes [35], we next examined whether Zeb1 repressed T-cadherin promoter activity. Exogenously expressed Zeb1 decreased wild-type (5′-CACCTG-3′) but not mutated E2-box-type (5′-AAATTT-3′) T-cadherin promoter activity in Cos7 (Fig. 1D). Notably, exogenous Zeb1 expression reduced T-cadherin mRNA in OCUG-1 cell (Fig. 1E). Moreover, exogenously expressed

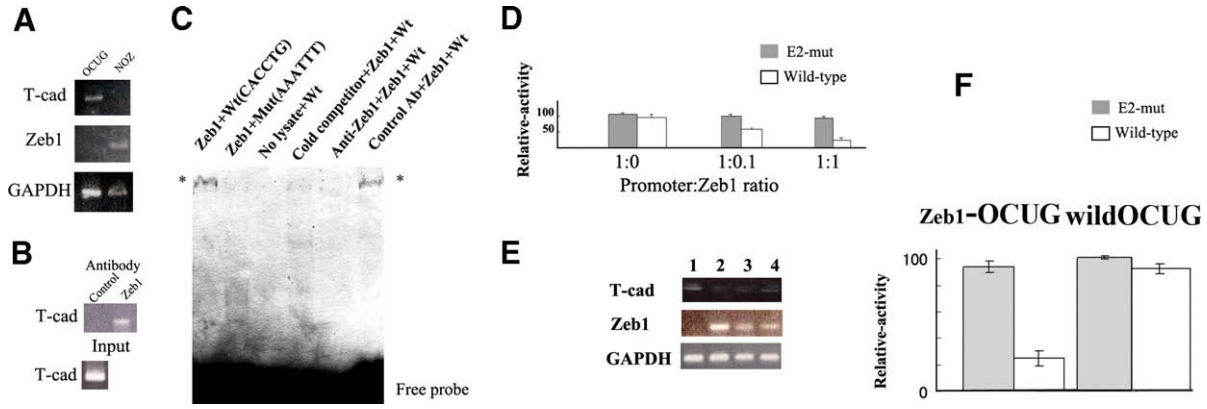

Fig. 1. Zeb1 directly associated with T-cadherin promoter and repressed its activity in E2-box element-dependent fashion. (A) RT-PCR demonstrated that OCUG-1 gallbladder cancer cells expressed T-cadherin mRNA but non-detectable levels of *Zeb1* mRNA, while NOZ gallbladder cancer cells expressed *Zeb1* mRNA but non-detectable levels of T-cadherin mRNA. (B) A chromatin immunoprecipitation experiment demonstrated that endogenous Zeb1 bound to the promoter region of T-cadherin. (C) Electrophoretic mobility shift assay (EMSA). Nuclear extracts were incubated with digoxigenin-11-ddUTP–labeled probe (−683 to −602), which had the wild-type E2-box 5′-CACCTG-3′ (Wt), or mutated 5′-AAATTT-3′ sequence (Mut), and were resolved by gel electrophoresis. Unlabeled competitor at 50-fold molar excess (Cold competitor), anti-Zeb1, or control antibody, was added to the binding reactions 5 min before the labeled probe and allowed to incubate at room temperature. The anti-Zeb1 antibody used in the present study disrupted Zeb1 binding to the probe. Asterisk (*) indicates the retarded DNA–protein complex. (D) Zeb1 repressed the activity of T-cadherin promoter in E2-box element- and dose-dependent fashion in Cos7 cells. A 5 μg portion of wild-type or E2-box-mutated T-cadherin promoter vector was co-transfected with Zeb1-expression vector (0, 0.5, or 5 μg). Significant, dose-dependent repression of wild-type T-cadherin promoter by Zeb1 was observed. Note that E2-box-mutated promoter activity was not affected by Zeb1. Promoter activity was measured using a GreatEscApeTM SEAP fluorescence detection kit (BD Biosciences Clontech) in supernatant of cell cultures in triplicate. Percent ratio of activity with 5 μg-wild-type T-cadherin promoter transfectant, substituted by the activity of control non-transfected Cos7 cells, was assigned a value of 100 (%). Values are means ± S.D. ($n = 3$). (E) Exogenous Zeb1 expression reduced T-cadherin mRNA level in OCUG-1 cells. Independent Zeb1-expressing OCUG-1 cells clones were obtained by transfection with pTarget-T expression vector containing Zeb1 cDNA after G418 selection. All three independent Zeb1-expressing OCUG-1 cells expressed little T-cadherin mRNA (lanes 2, 3, and 4). An OCUG-1 cell clone, which was transfected with empty vector, expressed T-cadherin, but not Zeb1, as found in the original cells (lane 1). (F) Promoter assay using intact OCUG-1 (indicated as wildOCUG) and Zeb1-expressing OCUG-1 (Zeb1-OCUG). The activity of T-cadherin promoter with wild-type E2-box element was significantly inhibited in Zeb1-expressing OCUG cells. Representative results obtained using a transfectant clone are shown. Similar results were obtained using other transfectants.

Zeb1 significantly decreased wild-type (5′-CACCTG-3′) but not mutated E2-box-type (5′-AAATTT-3′) T-cadherin promoter activity in OCUG-1 (Fig. 1F).

These findings indicate that Zeb1 repressed T-cadherin promoter activity through the E2-box sequence.

3.2. T-cadherin is a negative regulator of gallbladder cancer invasion

Zeb1-mediated repression of various genes has been found to induce EMT in cancer cells. We therefore next examined whether down-regulation of T-cadherin altered the malignant phenotype of gallbladder cancer. We treated T-cadherin-expressing OCUG-1 cells with T-cadherin-specific and control siRNA. Significant silencing of T-cadherin expression was observed in OCUG-1 cells with use of T-cadherin-specific siRNA but not control siRNA (representative findings shown in Fig. 2A and B). Interestingly, siRNA-silencing of T-cadherin increased collagen-gel invasive activity of OCUG-1 cells without altering cell growth (Fig. 2C and D).

3.3. Effects of Zeb1and T-cadherin on cell phenotype and invasion activity of cultured gallbladder cancer cells

We subsequently examined whether exogenous T-cadherin expression or silencing of Zeb1 expression altered the phenotype and invasive activity of NOZ cells. We generated a tetracycline-induced T-cadherin expression system using T-cadherin-negative NOZ cells. We established three independent clones (#2, #5, and #6), in which T-cadherin expression was regulated by tetracycline. In these clones, T-cadherin transcript was induced after 24 h by the addition of 1 μg/ml tetracycline (Supplementary Fig. S2). Silencing of Zeb1 expression was also performed in NOZ cells with use of Zeb1-specific siRNA but not control siRNA (Supplementary Fig. S3).

We found epithelial cell-like cell clusters in the case of both T-cadherin-expressing and Zeb1-silencing NOZ cells, whereas intact NOZ cells exhibited mesenchymal cell feature (Fig. 3A). Notably,

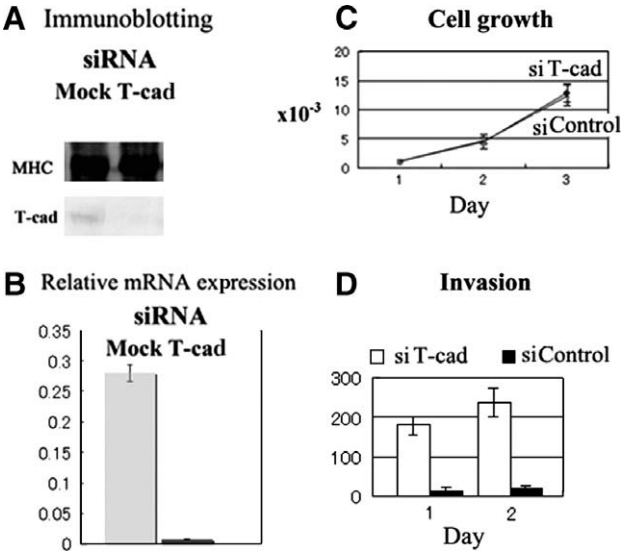

Fig. 2. siRNA silencing of T-cadherin increased gallbladder cancer cell invasion without affecting cell growth. T-cadherin was significantly down-regulated with use of specific siRNA for T-cadherin but not control siRNA (indicated by MOCK) in OCUG-1 cells (A: Western-immunoblotting, B: real-time PCR, $P < 0.01$). There was no significant difference in growth between T-cadherin-specific siRNA-treated and control OCUG-1 cells (C). In contrast, T-cadherin-negative OCUG-1 cells exhibited much weaker invasive activity than control cells (D, $P < 0.01$). Bars represent standard errors of the mean.

silencing of Zeb1 increased E-cadherin and T-cadherin expression in NOZ cells (Fig. 3B). In contrast, silencing of Zeb1 decreased N-cadherin expression. Both T-cadherin expression and Zeb1 silencing significantly reduced collagen gel invasive activity of NOZ cells (Fig. 3C).

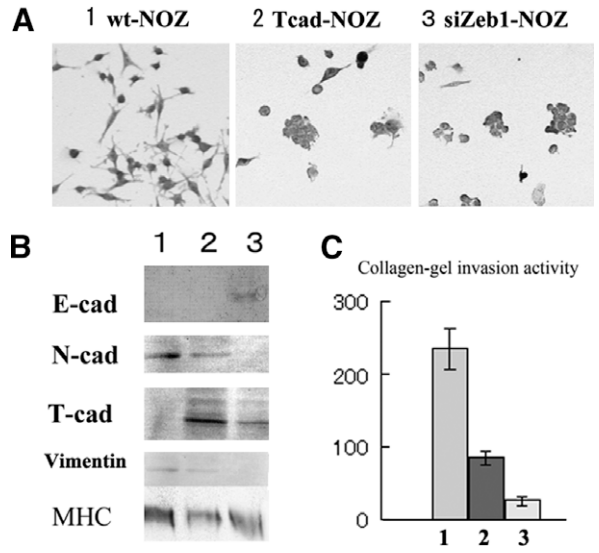

Fig. 3. Zeb1 and T-cadherin modulated morphological phenotype and invasive activity of NOZ gallbladder cancer cells. (A) Intact NOZ cells exhibited lymphoid and spindle fibroblast-like morphology, a feature of mesenchymal phenotype. Monolayer clusters with epithelial cell morphology were observed in the case of T-cadherin-expressing and Zeb1-silencing NOZ cells. Cells were with Giemsa-stained (original magnification, ×50). (B) Western-immunoblotting. Modulation of E-cadherin and N-cadherin expression was found in Zeb1-silencing NOZ cells, but not in T-cadherin-expressing NOZ cells. (C) Representative result of collagen gel invasion assay at Day1. Enforced T-cadherin expression and silencing of Zeb1 resulted in significant down-regulation of invasive activity of NOZ cells ($P < 0.01$). Values are means ± S.D. ($n = 3$).

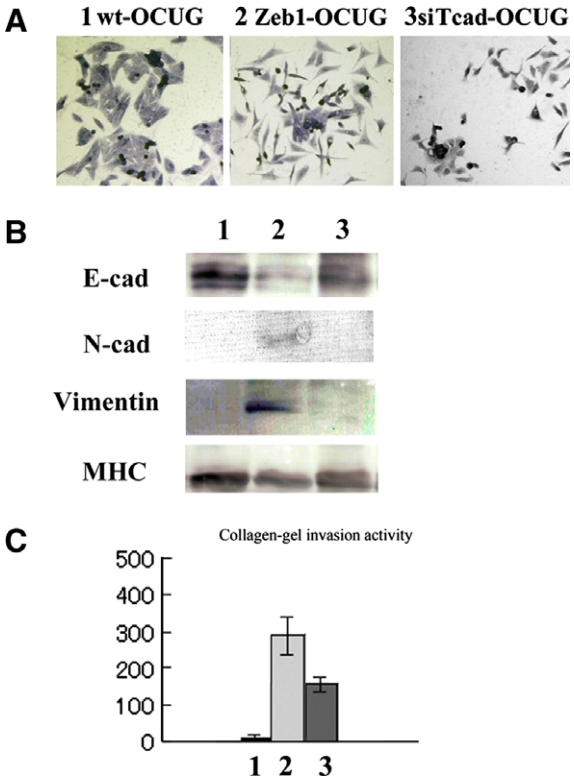

Fig. 4. Zeb1 and T-cadherin modulated morphological phenotype and invasive activity of OCUG-1 gallbladder cancer cells. (A) Intact OCUG-1 cells exhibited monolayer clusters with epithelial cell features. Notably, many fibroblastic spindle cells were observed in the case of Zeb1-expressing and T-cadherin-silencing OCUG-1 cells. Cells were with Giemsa-stained (original magnification, ×50). (B) Western-immunoblotting. Modulation of E-cadherin, N-cadherin, and vimentin expression was found in Zeb1-expressing and T-cadherin-silencing OCUG-1 cells. (C) Representative result of collagen gel invasion assay at Day1. Exogenous Zeb1 expression and down-regulation of T-cadherin expression significantly increased the invasive activity of OCUG-1 cells ($P < 0.01$). Values are means ± S.D. ($n = 3$).

We further asked whether exogenous Zeb1 expression altered the phenotype and invasive activity of OCUG-1 cells as observed for T-cadherin-silencing OCUG cells. As demonstrated in Fig. 4A, wild-type OCUG-1 displayed the epithelial phenotype; however, a clear morphological change, involving a spindle mesenchymal cell phenotype, was induced by enforced Zeb1 expression and silencing of T-cadherin expression. Down-regulation of E-cadherin, in contrast to up-regulation of N-cadherin and vimentin, was demonstrated in Zeb1-expressing OCUG-1 cells (Fig. 4B). Zeb1 expression significantly increased collagen-gel invasive activity of OCUG-1 cells (Fig. 4C). Notably, the invasive activity of Zeb1 expressing OCUG-1 cells was much high than that of T-cadherin-negative OCUG-1 cells.

These findings, together with the result of the promoter assay, suggest that Zeb1-mediated silencing of T-cadherin facilitates the invasion of gallbladder cancer cells in vitro. Notably, neither enforced T-cadherin expression in NOZ cells nor down-regulation of T-cadherin in OCUG-1 cells affected the E-cadherin or N-cadherin expression (Figs. 3B and 4B). Zeb1-mediated down-regulation of T-cadherin might independently contribute to gallbladder cancer invasion apart from the E-cadherin system.

3.4. Zeb1 and T-cadherin expression on gallbladder cancer cells

It is important to know the Zeb1 and T-cadherin expression in gallbladder cancer tissues. We could find no previous report that immunohistochemically examined T-cadherin and/or Zeb1 expression in gallbladder cancer tissue specimens. Therefore, immunohistochemical staining was carried out using archival pathological gallbladder cancer specimens.

Representative results are demonstrated in Fig. 5. With use of anti-Zeb1 antibody, immunoreactivity was found in 23 of 30 invasive gallbladder cancer tissue specimens, almost exclusively at sites of cancer invasion. Notably, Zeb1 was little expressed in non-cancerous epithelium.

By contrast, T-cadherin expression was found in 13 of 30 gallbladder cancer tissues at non-invasive foci. Preadsorption of antibodies with immunized antigen completely eliminated immunoreactivity, verifying antibody specificity. No staining was observed in tissues examined with control antibody.

These findings indicated that Zeb1 was overexpressed at sites of cancer invasion, where T-cadherin was not expressed.

4. Discussion

Loss of T-cadherin expression has been reported in many malignant tumors [14,22–25,36–38]. Although many studies, including our own, have demonstrated hypermethylation of T-cadherin promoter in various cancers, they did not demonstrate that aberrant methylation of the promoter region was solely responsible for silencing of T-cadherin.

Takahashi et al., using genomic DNA from whole frozen tissues, reported that aberrant methylation of the T-cadherin promoter was found in 44% of gallbladder cancer specimens, most of which were in invasive stage (45 of 50 cases) [39]. However, in the present study, we found no significant T-cadherin expression in invasive foci of 30 gallbladder cancer specimens. It is thus likely that molecular mechanisms other than aberrant methylation participate in T-cadherin silencing.

The T-cadherin promoter segment, which spans −1 to −682, contains a typical canonical E2-box, 5′-CACCTG-3′, for binding of Zeb1. It is known that Zeb1 represses transcription through bind-

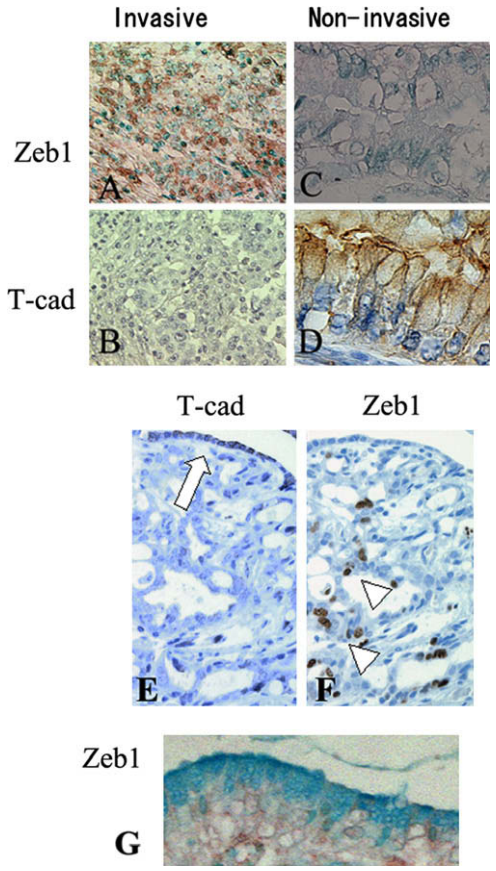

Fig. 5. Representative immunohistochemical staining of gallbladder cancer tissues. Zeb1 immunoreactivity was observed in invasive foci (A) but not in non-invasive foci (C) of gallbladder cancer. In contrast, T-cadherin expression was found in non-invasive foci (D) but not invasive foci in gallbladder cancer tissue specimens. Contrasting staining with anti-T-cadherin antibodies at non-invasive cancer (arrow) and anti-Zeb1 at invasive cancer (arrow head) in the same tissue specimens were shown in E and F, respectively. Notably, no significant immunoreactivity with anti-Zeb1 antibody was observed in non-cancerous epithelium of gallbladder bladder (G). Original magnifications, ×100 (E, F, and G), ×200 (A and B), and ×400 (C and D).

ing to the sequence 5′-CACCT-3′ [26,40–43]. Sekido et al. extensively examined the Zeb1 (delta-EF1)-binding sequence and found that Zeb1 binds strongly to 5′-CACCTG-3′ and 5′-CACCTT-3′, moderately to 5′-CACCTA-3′, but weakly or not at all to 5′-CACCTC-3′ [43].

The promoter segment has a canonical Zeb1 binding sequence, 5′-CACCTG-3′, but neither 5′-CACCT-3′ nor 5′-CACCTA-3′ sequence. The findings of EMSA in the present study showed that replacement of the 5′-CACCTG-3′ sequence with the 5′-AAATTT-3′ sequence markedly inhibited Zeb1 binding to the T-cadherin promoter region. These data indicates that the 5′-CACCTG-3′ sequence of the T-cadherin promoter is essential for Zeb1 regulation.

Exogenous Zeb1 expression decreased T-cadherin expression in OCUG-1 gallbladder cancer cells. By contrast, siRNA silencing of Zeb1 increased T-cadherin expression in NOZ cells. Moreover, while expression of Zeb1 was found at sites of cancer invasion, that of T-cadherin was found at non-invasive foci also suggesting that over-expression of Zeb1 might repress T-cadherin expression in gallbladder cancer.

Over-expression of Zeb1 is also found in colorectal [44] and lung cancer [45], in which expression of T-cadherin is suppressed [23,24,36]. These findings suggest that Zeb1 may repress T-cadherin expression in various cancers, as demonstrated in gallbladder cancer.

Recent studies have highlighted Zeb1 as one of the key molecules responsible for EMT [12] and the invasive activity of cancer cells [46]. Cadherin switching followed by activation of cancer invasion is often found in EMT [47]. It is known that E-cadherin is down-regulated while expression of N-cadherin is increased during cancer progression [47]. T-cadherin is also down-regulated during the progression of various types of cancer.

OCUG-1 cells expressed E-cadherin but not N-cadherin, while NOZ cells expressed N-cadherin but not E-cadherin. OCUG-1 cells proliferated in monolayer sheets with epithelial cell phenotype, whereas NOZ cells were spindle-shaped, a feature of mesenchymal cells. In the present study, silencing of T-cadherin resulted in morphological change from epithelial to mesenchymal-like cells and decreased collagen-gel invasive activity in OCUG-1 cells. By contrast, exogenous T-cadherin expression reduced the collagen-gel invasion activity of NOZ cells. Lee et al also reported that restoration of T-cadherin markedly inhibits breast cancer cell invasion [48]. These findings together suggest that Zeb1-mediated T-cadherin suppression may play a role in EMT and could increase cancer cell invasion.

T-cadherin is a glycosyl phosphatidyl inositol (GPI)-linked surface membrane protein [13], and lacks the cytoplasmic domain required for the homophilic binding activity of classical cadherins [16]. It has been thought that T-cadherin does not mediate cell-cell adhesion, as found for E-cadherin in physiological conditions. Consistent with this, a recent study based on NMR structural examination clearly demonstrated that T-cadherin is monomeric in both the presence and absence of calcium [21], and may thus lack homophilic cell-cell adhesive activity. These studies suggest that a novel molecular mechanism not involving intracellular adhesion may be responsible for cancer invasion followed by loss of T-cadherin. We are now examining the molecular profile of gallbladder cancer cells with or without induction of T-cadherin using a tetracycline-induced T-cadherin expression system.

In summary, the present study yielded three findings. First, Zeb1 was overexpressed in many gallbladder cancers, especially at sites of cancer invasion. Second, Zeb1 participated in the silencing of T-cadherin through E2-box elements of the T-cadherin promoter. Third, T-cadherin may be involved in EMT and a negative regulator of gallbladder cancer cells.

Acknowledgements

We thank Ms. Naoyo Nakamura (Department of Pathology, Kochi Medical School) and Ms. Rumi Matsumura (Division of Molecular Biology, Kochi Medical School) for their skilled assistance. This study was supported by grants from the Ministry of Education of Japan (KAKEN 12670165, 13670177, 17590270) and The Kochi University President's Discretionary Grant.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.12.042.

References

[1] Gourgiotis, S., Kocher, H.M., Solaini, L., Yarollahi, A., Tsiambas, E. and Salemis, N.S. (2008) Gallbladder Cancer 196, 252–264.
[2] Schottenfeld, D. and Beebe-Dimmer, J. (2006) Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. CA Cancer J. Clin. 56, 69–83.
[3] Zatonski, W.A., Lowenfels, A.B., Boyle, P., Maisonneuve, P., Bueno De Mesquita, H.B., Ghadirian, P., Jain, M., Przewozniak, K., Baghurst, P., Moerman, C.J., Simard, A., Howe, G.R., McMichael, A.J., Hsieh, C.C. and Walker, A.M. (1997) Epidemiologic aspects of gallbladder cancer: a case–control study of the SEARCH Program of the International Agency for Research on Cancer. J. Natl. Cancer Inst. 89, 1132–1138.

[4] Takagi, S., Naito, E., Yamanouchi, H., Ohtsuka, H., Kominami, R. and Yamamoto, M. (1994) Mutation of the p53 gene in gallbladder cancer. Tohoku J. Exp. Med. 172, 283–289.
[5] Wee, A., The, M. and Raju, G.C. (1994) Clinical importance of p53 protein in gallbladder carcinoma and its precursor lesions. J. Clin. Pathol. 47, 453–456.
[6] Diamantis, I., Karamitopoulou, E., Perentes, E. and Zimmermann, A. (1995) P53 protein immunoreactivity in extrahepatic bile duct and gallbladder cancer: correlation with tumor grade and survival. Hepatology 22, 774–779.
[7] Malats, N., Porta, M., Pinol, J.L., Corominas, J.M., Rifa, J. and Real, F.X. (1995) Ki-ras mutations as a prognostic factor in extrahepatic bile system cancer. PANK-ras I Project Investigators. J. Clin. Oncol. 13, 1679–1686.
[8] Yoshida, S., Todoroki, T., Ichikawa, Y., Hanai, S., Suzuki, H., Hori, M., Fukao, K., Miwa, M. and Uchida, K. (1995) Mutations of p16Ink4/CDKN2 and p15Ink4B/MTS2 genes in biliary tract cancers. Cancer Res. 55, 2756–2760.
[9] Thiery, J.P. (2003) Epithelial-mesenchymal transitions in development and pathologies. Curr. Opin. Cell Biol. 15, 740–746.
[10] Huber, M.A., Kraut, N. and Beug, H. (2005) Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr. Opin. Cell Biol. 17, 548–558.
[11] Peinado, H., Olmeda, D. and Cano, A. (2007) Snail, ZEB and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 7, 415–428.
[12] Liu, Y., El-Naggar, S., Darling, D.S., Higashi, Y. and Dean, D.C. (2008) Zeb1 links epithelial-mesenchymal transition and cellular senescence. Development 135, 579–588.
[13] Ranscht, B. and Dours-Zimmermann, M.T. (1991) T-cadherin, a novel cadherin cell adhesion molecule in the nervous system, lacks the conserved cytoplasmic region. Neuron 7, 391–402.
[14] Takeuchi, T., Liang, S.B., Matsuyoshi, N., Zhou, S., Miyachi, Y., Sonobe, H. and Ohtsuki, Y. (2002) Loss of T-cadherin (CDH13, H-cadherin) expression in cutaneous squamous cell carcinoma. Lab. Invest. 82, 1023–1029.
[15] Zhou, S., Matsuyoshi, N., Liang, S.B., Takeuchi, T., Ohtsuki, Y. and Miyachi, Y. (2002) Expression of T-cadherin in Basal keratinocytes of skin. J. Invest. Dermatol. 118, 1080–1084.
[16] Nagafuchi, A. and Takeichi, M. (1988) Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J. 7, 3679–3684.
[17] Takeuchi, T., Misaki, A., Liang, S.B., Tachibana, A., Hayashi, N., Sonobe, H. and Ohtsuki, Y. (2000) Expression of T-cadherin (CDH13, H-cadherin) in human brain and its characteristics as a negative growth regulator of EGF in neuroblastoma cells. J. Neurochem. 74, 1489–1497.
[18] Li, X., Massa, P.E., Hanidu, A., Peet, G.W., Aro, P., Savitt, A., Mische, S., Li, J. and Marcu, K.B. (2002) IKKalpha, IKKbeta, and NEMO/IKKgamma are each required for the NF-kappa B-mediated inflammatory response program. J. Biol. Chem. 277, 45129–45140.
[19] Huang, Z.Y., Wu, Y., Hedrick, N. and Gutmann, D.H. (2003) T-cadherin-mediated cell growth regulation involves G2 phase arrest and requires p21(CIP1/WAF1) expression. Mol. Cell. Biol. 23, 566–578.
[20] Ivanov, D., Philippova, M., Allenspach, R., Erne, P. and Resink, T. (2004) T-cadherin upregulation correlates with cell-cycle progression and promotes proliferation of vascular cells. Cardiovasc Res. 64, 132–143.
[21] Dames, S.A., Bang, E., Haüssinger, D., Ahrens, T., Engel, J. and Grzesiek, S. (2008) Insights into the low adhesive capacity of human T-cadherin from the NMR structure of its N-terminal extracellular domain. J. Biol. Chem. 283, 23485–23495.
[22] Lee, S.W. (1996) H-cadherin, a novel cadherin with growth inhibitory functions and diminished expression in human breast cancer. Nat. Med. 2, 776–782.
[23] Toyooka, K.O., Toyooka, S., Virmani, A.K., Sathyanarayana, U.G., Euhus, D.M., Gilcrease, M., Minna, J.D. and Gazdar, A.F. (2001) Loss of expression and aberrant methylation of the CDH13 (H-Cadherin) gene in breast and lung carcinomas. Cancer Res. 61, 4556–4560.
[24] Toyooka, S., Toyooka, K.O., Harada, K., Miyajima, K., Makarla, P., Sathyanarayana, U.G., Yin, J., Sato, F., Shivapurkar, N., Meltzer, S. and Gazdar, A.F. (2002) Aberrant methylation of the CDH13 (H-cadherin) promoter region in colorectal cancers and adenomas. Cancer Res. 62, 3382–3386.
[25] Takeuchi, T., Liang, S.B. and Ohtsuki, Y. (2002) Down-regulation of expression of a novel cadherin molecule, T-cadherin, in basal cell carcinoma of the skin. Mol. Carcinog. 35, 173–179.
[26] Genetta, T., Ruezinsky, D. and Kadesch, T. (1994) Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implication for B-cell specificity of the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. 14, 6153–6163.
[27] Bromhead, C., Miller, J.H. and McDonald, F.J. (2006) Regulation of T-cadherin by hormones, glucocorticoid and EGF. Gene 374, 58–67.
[28] Takeuchi, T., Adachi, Y., Sonobe, H., Furihata, M. and Ohtsuki, Y. (2006) A ubiquitin ligase, skeletrophin, is a negative regulator of melanoma invasion. Oncogene 25, 7059–7069.
[29] Darling, D.S., Stearman, R.P., Qi, Y., Qiu, M.S. and Feller, J.P. (2003) Expression of Zfhep/δEF1 protein in palate, neural progenitors, and differentiated neurons. Gene Exp. Patterns 3, 709–717.
[30] Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[31] Horiuchi, H., Kawamata, H., Fujimori, T. and Kuroda, Y. (2003) A MEK inhibitor (U0126) prolongs survival in nude mice bearing human gallbladder cancer cells with K-ras mutation: analysis in a novel orthotopic inoculation model. Int. J. Oncol. 23, 957–963.
[32] Tekant, Y., Davydova, J., Ramirez, P.J., Curiel, D.T., Vickers, S.M. and Yamamoto, M. (2005) Oncolytic adenoviral therapy in gallbladder carcinoma. Surgery 137, 527–535.
[33] Greene, F.L., Page, D.L., Fleming, I.D., Fritz, A.G., Balch, C.M., Haller, D.G. and Morrow, M. (2002) American Joint Committee on Cancer Staging Manual, sixth ed, Springer, New York. pp. 139–144.
[34] Philippova, M.P., Bochkov, V.N., Stambolsky, D.V., Tkachuk, V.A. and Resink, T.J. (1998) T-cadherin and signal-transducing molecules co-localize in caveolin-rich membrane domains of vascular smooth muscle cells. FEBS Lett. 429, 207–210.
[35] Chen, J., Yusuf, I., Andersen, H.M. and Fruman, D.A. (2006) FOXO transcription factors cooperate with delta EF1 to activate growth suppressive genes in B lymphocytes. J. Immunol. 176, 2711–2721.
[36] Sato, M., Mori, Y., Sakurada, A., Fujimura, S. and Horii, A. (1998) The H-cadherin (CDH13) gene is inactivated in human lung cancer. Hum. Genet. 103, 96–101 (Published erratum in Hum. Genet. 103 (1998) 532).
[37] Roman-Gomez, J., Castillejo, J.A., Jimenez, A., Cervantes, F., Boque, C., Hermosin, L., Leon, A., Grañena, A., Colomer, D., Heiniger, A. and Torres, A. (2003) Cadherin-13, a mediator of calcium-dependent cell-cell adhesion, is silenced by methylation in chronic myeloid leukemia and correlates with pretreatment risk profile and cytogenetic response to interferon alfa. J. Clin. Oncol. 21, 1472–1479.
[38] Sakai, M., Hibi, K., Koshikawa, K., Inoue, S., Takeda, S., Kaneko, T. and Nakao, A. (2004) Frequent promoter methylation and gene silencing of CDH13 in pancreatic cancer. Cancer Sci. 95, 588–591.
[39] Takahashi, T., Shivapurkar, N., Riquelme, E., Shigematsu, H., Reddy, J., Suzuki, M., Miyajima, K., Zhou, X., Bekele, B.N., Gazdar, A.F. and Wistuba, I.I. (2004) Aberrant promoter hypermethylation multiple genes in gallbladder carcinoma and chronic cholecystitis. Clin. Cancer Res. 10, 6126–6133.
[40] Kamachi, Y. and Kondoh, H. (1993) Overlapping positive and negative regulatory elements determine lens-specific activity of the δ1-crystallin enhancer. Mol. Cell. Biol. 13, 5206–5215.
[41] Franklin, A.J., Jelton, T.L., Shelton, K.D. and Magnuson, M.A. (1994) BZP, a novel serum-responsive zinc finger protein that inhibits gene transcription. Mol. Cell. Biol. 14, 6773–6788.
[42] Postigo, A.A. and Dean, D.C. (1997) ZEB, a vertebrate homologue of *Drosophila* Zfh-1, is a negative regulator of muscle differentiation. EMBO J. 16, 3935–3943.
[43] Sekido, R., Murai, K., Funahashi, J., Kamachi, Y., Fujisawa-Sehara, A., Nabeshima, Y. and Kondoh, H. (1994) The δ-crystallin enhancer-binding protein δEF1 is a repressor of E2-box-mediated gene activation. Mol. Cell. Biol. 14, 5692–5700.
[44] Aigner, K., Dampier, B., Descovich, L., Mikula, M., Sultan, A., Schreiber, M., Mikulits, W., Brabletz, T., Strand, D., Obrist, P., Sommergruber, W., Schweifer, N., Wernitznig, A., Beug, H., Foisner, R. and Eger, A. (2007) The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. Oncogene 26, 6979–6988.
[45] Dohadwala, M., Yang, S.C., Luo, J., Sharma, S., Batra, R.K., Huang, M., Lin, Y., Goodglick, L., Krysan, K., Fishbein, M.C., Hong, L., Lai, C., Cameron, R.B., Gemmill, R.M., Drabkin, H.A. and Dubinett, S.M. (2006) Cyclooxygenase-2-dependent regulation of E-cadherin: prostaglandin E(2) induces transcriptional repressors ZEB1 and snail in non-small cell lung cancer. Cancer Res. 66, 5338–5345.
[46] Sarrió, D., Rodriguez-Pinilla, S.M., Hardisson, D., Cano, A., Moreno-Bueno, G. and Palacios, J. (2008) Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res. 68, 989–997.
[47] Stemmler, M.P. (2008) Cadherins in development and cancer. Mol. Biosyst. 4, 835–850.
[48] Lee, S.W., Reimer, C.L., Campbell, D.B., Cheresh, P., Duda, R.B. and Kocher, O. (1998) H-cadherin expression inhibits in vitro invasiveness and tumor formation in vivo. Carcinogenesis 19, 1157–1159.